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(21) International Application Number: PCT/US95/04975 (22) International Filing Date: 21 April 1995 (21.04.95) (30) Priority Data: 08/233,496 22 April 1994 (22.04.94) US (71) Applicant: SLOAN-KETTERING INSTITUTE FOR CAN- CER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US). (72) Inventors: NIKOLIC-ZUGIC, Janko; Apartment 12P, 504 East 63rd Street, New York, NY 10021 (US). DYALL, Rubendra; Apartment 2A, 529 East 13th Street, New York, NY 10009 (US). (74) Agent: WHITE, John, P.; Cooper & Dunham L.L.P., 1185 Avenue of the Americas, New York, NY 10036 (US).		(81) Designated States: AU, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: INDUCTION OF CYTOTOXIC T LYMPHOCYTES (CTL) USING ANTIGENIC PEPTIDES AND A SUITABLE ADJUVANT (57) Abstract The present invention provides a method of treating a tumor-bearing subject, a method of inducing cytotoxic antitumor T lymphocytes, a method of treating pathogenic disease, and a method of vaccinating a subject comprising administering an MHC Class I restricted, 8-12 amino acid residue antigenic peptide in combination with a suitable adjuvant, such as TiterMax.		

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INDUCTION OF CYTOTOXIC T LYMPHOCYTES (CTL) USING
ANTIGENIC PEPTIDES AND A SUITABLE ADJUVANT

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Throughout this application, various publications are referenced by arabic numerals in brackets. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

15 BACKGROUND OF INVENTION

CD8⁺ major histocompatibility complex (MHC) class I molecule - restricted cytotoxic T lymphocytes (CTL) play a major role in mounting a specific immune response against intracellular pathogens. A successful strategy to induce specific CTL responses against these pathogens would greatly enhance the ability to control various viral, parasitic and bacterial diseases by vaccination. Recently, several groups reported successful induction of CTLs using multiple doses of 15-16 amino acid long synthetic peptides emulsified in the incomplete Freund's adjuvant. However, general applicability of this method was not tested.

30 This invention discloses a simple and highly reproducible method of generating CTL activity against eight tested CTL determinants by a single dose of peptide immunization, in two different strains of mice. The immunization strategy combines the optimal MHC class I restricted peptide determinant (octa- or nonameric peptide) with a synthetic, commercially available adjuvant TiterMax®. CTL activity elicited in this fashion is mediated by CD8⁺ cells, and is physiologically relevant: peptide-elicited CTLs are capable of lysing

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target cells that endogenously synthesize and process the determinant, including virally infected targets. The advantages of this method over previously published methods are that : (i) 8-10 amino acid long peptides, optimal for class I binding, are very effective; (ii) a single injection is sufficient to induce a strong response; and (iii) the method is generally applicable, since it produced results with six H-2^b restricted peptides and two H-2^d restricted peptides. Because of the small peptide size, the chance of autoimmune and allergic complications is greatly reduced and the cost is also reduced.

Most importantly, this method can be used to elicit potent anti-tumor CTLs. In fact, in experiments, peptide immunization induced CTLs that protect animals against otherwise lethal tumors. Thus, peptide treatment and vaccination has considerable diagnostic, preventive and therapeutic potential.

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SUMMARY OF INVENTION

5 The present invention provides a method of treating a subject with a tumor which comprises administering to the subject an effective amount of a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the tumor. Further, the suitable adjuvant is TiterMax®.

10

In addition, the present invention provides a method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby induce anti-tumor cytotoxic T lymphocytes in the subject. Further, the present invention provides for an anti-tumor cytotoxic T lymphocyte.

20

In addition, the present invention provides a method of treating a subject with a pathogenic disease which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the pathogenic disease.

25

In addition, the present invention provides a method of vaccinating a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby vaccinate the subject.

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35

BRIEF DESCRIPTION OF FIGURES

- 5 **Figure 1A-1B.** Surface phenotype of peptide-induced CTLs. HSV peptide-induced CTL lines were phenotyped for the expression of CD8⁺ and CD4⁺ (Figure 1A) and TCR (Figure 1B) as described in Methods, and expressed as contour plots (Figure 1A) or open histograms (Figure 1B). Filled histogram in (Figure 1B) represents the fluorescence of control-stained cells. Similar results were obtained with CTL lines induced by five different peptides.
- 10
- 15 **Figure 2.** CTL activity of spleen cells obtained by immunization with HSV pep/TM, TM or pep. Immunization, restimulation and CTL assay were performed as described in Methods. CTL activity was tested on HSV peptide -
- 20 coated EL-4 cells or control EL-4 cells. The lysis of the latter was <5%.
- 25 **Figure 3.** K^d-restricted CTLs induced by pep/TM. B6D2 F₁ mice were immunized by SVT/TM (positive control), LLO/TM or p60/TM, restimulated in vitro, and tested for cytolytic activity against EL-4 or P815 cells in the presence (solid symbols) or the absence (open symbols) of
- 30 corresponding peptides. Results from primary cultures are displayed.
- 35 **Figure 4A.** E.G7 tumor is not rejected by lightly irradiated animals. B6 mice (2/group) were irradiated with 4 Gy and inoculated subcutaneously in the flank with either 2 x 10⁷ (triangles) or 2 x 10⁶ (circles) E.G7

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cells. Tumor growth and status was monitored daily, and is expressed as tumor diameter (mm), obtained by multiplying two orthogonal measurements of the tumor using calipers, and by extracting a square root from this value.

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Figure 4B.

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Vaccination with pep/TM protects against tumor growth. B6 animals were immunized with OVA/TM on day 0. Seven days latter, mice were irradiated and injected with E.G7 cells (7×10^6 /recipient). Tumor growth was scored as described in Figure 4A.

15

Figure 5.

20

Cocktail immunization can elicit CTL activity against each of the peptides in the cocktail mixture. B6 mice were immunized with a cocktail containing 5 μ g each of OVA, FLU and SVT peptides in TM. CTL activity of split cultures restimulated on each of the three peptides is shown on EL-4 targets with (filled symbols) and without (open symbols) peptide. Representative results from three experiments are shown. FLU-triangles; OVA-circles; SVT-diamonds.

25

Figure 6.

30

CD4⁺ dependence of pep/TM responses. B6 mice were treated with successive injections of purified GK 1.5 mAb (circles) or saline (triangles), and immunized with the SVT peptide. CTL activity was tested after in vitro restimulation against EL-4 target cells in the presence (filled symbols) or absence (open symbols) of the SVT peptide.

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DETAILED DESCRIPTION OF THE INVENTION

5 The present invention provides a method of treating a subject with a tumor which comprises administering to the subject an effective amount of a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the tumor.

10 In one embodiment the amino acid antigenic peptide is a small peptide sequence. In another embodiment the amino acid antigenic sequence is no more than 15 amino acids. In the preferred embodiment the amino acid sequence is 8-10 amino acids.

15 The "MHC class I restricted 8-12 amino acid antigenic peptide" is defined herein as a 8-12 amino acid sequence which when administered to the subject induces cytotoxic T lymphocytes (CTL) via MHC class I molecules in combination with a suitable adjuvant.

20 In one embodiment the suitable adjuvant consists of, or is a combination with, a metabolizable oil, squalene, or a block copolymer. In the preferred embodiment the suitable adjuvant is TiterMax® or "TITERMAX" (Vaxcel™, Inc.). More specifically, TiterMax® consists of a block copolymer CRL-8941, microparticulate silica coated with CRL-8941, sorbitan monooleate 80 and squalene.

30 Further, the effective amount the MHC class I restricted 8-12 amino acid antigenic peptide in combination with the effective amount of a suitable adjuvant is administered in combination with a second anti-tumor therapy.

35 As defined herein "a second anti-tumor therapy" is any therapy which is employed to treat a subject with a tumor. For example, therapies include but are not

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limited to: irradiation, cytostatic or chemotherapy. Chemotherapeutic agents, include but are not limited to: alkylating agents, i.e. nitrogen mustards, ethylenimines and methylemelamines, alkyl sulfonates, nitrosoureas, and triazenes. Further chemotherapeutic agents include antimetabolites, i.e. folic acid analogs, pyrimidine analogs, purine analogs and related inhibitors.

Further chemotherapeutic agents include natural products, i.e. vinca alkaloids, epipodophyllotoxins, antibiotics, enzymes, biological response modifiers. Further, chemotherapeutic agents include miscellaneous Agents, i.e. platinum coordination complexes, anthracenedione, substituted urea, methyl hydrazine derivative, and adrenocortical suppressant. Lastly, chemotherapeutic agents include hormones and antagonists, i.e. adrenocorticosteroids, progestins, estrogens, antiestrogen, androgens, antiandrogen, and gonadotropin-releasing hormone analog.

As defined herein "tumor" includes but is not limited to: sarcomas, carcinomas, fibrosarcoma, osteosarcoma, chondrosarcoma, neuroblastoma, retinoblastoma, B cell lymphoma, myeloblastic leukemia, and lymphatic leukemia.

Further, it is well known in the art how to determine the antigen or epitope of the CTL for a given tumor and the effective amount of the MHC Class I restricted 8-12 amino acid antigenic peptide corresponding to the tumor which is administered to the subject. Synthetically generating the peptide for the purpose of inducing and testing CTL responses are known to one skilled in the art [4]. For example, the HLA-B18 and HLA-B35 restricted epitope from the CMV may be employed.

Further, taking advantage of a high level bacterial expression vector, the regularity of exonuclease III DNA

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degradation, and rapid alkali hydrolysis, the CTL antigen can be located within cloned genes. Exonuclease III degrades DNA at roughly 200 nucleotides/min and only from a blunt or 5' overhang terminal. Thus, a large panel of
5 tightly nested deletions in the 3'- end of a gene may be constructed within an inducible prokaryotic expression vector. After IPTG-mediated induction of transcription, vector, derived protein is expressed at high levels for several hours and eventually constitutes approximately
10 one-third of the protein in these bacteria. This is adequate purity for the generation of targeting peptides from alkali digests of whole or lysed bacteria [3].

Further, E. coli over-expressing the cancer cell protein
15 of interest of lysates derived from such cultures may also be digested by alkali hydrolysis to generate targeting peptide. The location of the epitope may then be determined from a panel of Escherichia coli clones expressing various 3'- truncated forms of the gene. The
20 general applicability of this approach was demonstrated by screening two genes from the common pathogen, human CMV for two HLA class I-restricted epitopes. This method requires limited information about the target Antigen and the restricting MHC to rapidly and precisely localize CTL
25 epitopes [4].

For example, the antigenic peptides for MAGE which are present in melanoma, breast and bladder cancer are: EVDPIGHLY, EADPTGHSY, EVVPISHLY (SEQ. ID. NOS. 1-3).
30 Further, specific antigenic peptides for tumor cells are known to one skilled in the art.

In addition, this invention provides a method of inducing cytotoxic T lymphocytes in a subject which comprises
35 administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of "TITERMAX" so as

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to thereby induce cytotoxic T lymphocytes in the subject.

Further, the cytotoxic T lymphocytes is a anti-tumor cytotoxic T lymphocytes. As defined herein "anti-tumor
5 cytotoxic T lymphocytes" are CTL's specifically induced by an antigen, which is associated with a tumor or is present on tumor cells.

Further, the method of treating a subject with a tumor
10 may be with a plurality of antigenic peptides which are administered in combination with an effective amount of the suitable adjuvant.

In addition, this invention provides a method of treating
15 a subject with a pathogenic disease which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the
20 pathogenic disease.

In one embodiment the pathogenic disease is bacterial. In a another embodiment the pathogenic disease is parasitic. In another embodiment the pathogenic disease
25 is viral.

Further, bacterial diseases include, but are not limited to: Gram negative bacilli, such as Salmonella; Spirochetes; Gram positive cocci, such as, Staphylococcus
30 aureus, Streptococcus; Gram negative cocci, such as Neisseria gonorrhoea; Gram positive bacilli, such as Escherichia coli; and Gram negative bacilli; Acid fast bacilli.

Further, parasitic diseases include, but are not limited to: protozoan infections, such as Leishmaniasis, Trichomoniasis, Trypanosomiasis, Malaria, Amebiasis,
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Balantidiasis, and Giardiasis; and metazoan infections, such as, Hookworm, Trichinosis.

Further, viral diseases include, but are not limited to:

5 Human Immunodeficiency Virus, Herpesvirus, Varicella-Zoster Virus, Cytomegalovirus, Epstein-Barr Virus, Hepatitis B, Papillomavirus, Influenza, and Respiratory Syncytial Virus, and Simian Virus 40 (SVT).

10 Further, the present invention includes, but is not limited to, MHC class I restricted 8-12 amino acid antigenic peptides consisting of: EVDPIGHLY, EADPTGHSY, EVVPISHLY, EIRSLYNPV, PLTSCNTSV, GYKDGNEYI, KYGVSVQDI, SIINFEKL, RGYVYQGL, FAPGNYPAL, VVYDFLKCL, SSIEFARL,

15 ASNENMETM, GILGFVFPPL, LLFGYPVYV, ILKEPVHGV, KLGEFYNQMM, IAGNSAYEYV, FLASDFFPSV (SEQ. ID. NOS. 1-19).

Further, this invention provides for a plurality of antigenic peptides which can be administered in

20 combination with an effective amount of the suitable adjuvant.

In addition, this invention provides a method of vaccinating a subject which comprises administering to

25 the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby vaccinate the subject.

30 As defined in this invention, the word "vaccine" is an antigen source for activating an immune responses against established tumors or pathogenic diseases, and thus for prophylactic and preventative immunization.

35 Further, the method of vaccinating a subject may be with a plurality of antigenic peptides which are administered in combination with an effective amount of the suitable

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adjuvant as hereinabove described.

5 In addition, the present invention provides, a method of treating a subject with an auto-immune disease, which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide, in combination with an effective amount of a suitable adjuvant, so as to thereby treat the subject with the auto-immune disease.

10

As defined herein "auto-immune" diseases include, but are not limited to: Hashimoto's Thyroiditis, Pernicious Anemia, Addison's disease, Goodpasture's syndrome, male infertility, Multiple Sclerosis, Idiopathic leucopenia, 15 Ulcerative colitis, Rheumatoid arthritis, Scleroderma, Systemic Lupus Erythematosus. Other immune disorders include graft vs. host rejection and immunoincompetent subjects.

20 In addition, the present invention provides a method of assaying the MHC class I restricted antigenic peptide of a tumor of a subject by contacting the tumor of a subject with a panel of cytotoxic T lymphocytes with known MHC class I restricted 8-12 antigenic peptides so as to assay 25 the MHC class I restricted antigenic peptide of a tumor.

In addition, the present invention provides, a method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective 30 amount of a MHC class I restricted amino acid antigenic peptide in combination with an effective amount of "TITERMAX" so as to thereby induce cytotoxic T lymphocytes in the subject.

35 Further, the method of inducing cytotoxic T lymphocytes in a subject may be with a plurality of antigenic peptides which are administered in combination with an

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effective amount of the suitable adjuvant. Further, the antigenic peptides have hereinabove been described.

5 In addition, this invention provides, a kit for inducing cytotoxic T lymphocytes in a subject which comprises a suitable amount of MHC class I restricted 8-12 amino acid antigenic peptide and a suitable adjuvant. Further, the suitable adjuvant is "TITERMAX". Further, the antigenic peptides have been hereinabove described.

10

The kit may include, but is not limited to: MHC class I restricted 8-12 amino acid antigenic peptide in saline or other suitable fluid as hereinabove described (50 ng - 50 μ g); a block copolymer, such as CRL89-41 bonded to a the surface of a silica particles; water-in-oil emulsion containing a metabolizable non-toxic oil; squalene; and plastic syringes or other means to prepare suitable adjuvant for emulsification. Further, reagents include but are not limited to, demulsifying agents, such as SDS or other acrylamide gels.

20

As defined herein the "subject" may be a human, monkey, dog, cat, rabbit, horse, cow, chicken and rodent. In the preferred embodiment the subject is a human. In another embodiment, the subject is a rodent, more specifically a mouse.

25

As used herein administration means a method of administering to a subject. Such methods are well known to those skilled in the art and include, but are not limited to, administration topically, parenterally, orally, intravenously, intradermally, intranasally, intratumorally, intratracheal, intramuscularly, subcutaneously, or by catheter. Administration of the agent may be effected continuously or intermittently such that the therapeutic agent in the subject is effective to modulate or treat the neoplastic cell or tissue.

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Further, in the present invention booster shots, which are defined as shots after the initial administration, are not required. However, suitable regimes for initial administration and booster shots are variable, but are typified by an initial administration followed by repeated doses at one or more hour or day intervals by a subsequent injection or other administration may be employed.

10 In addition, this invention provides a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with a suitable adjuvant which may be formulated into the therapeutic composition so as to be neutralized pharmaceutically in an acceptable salt forms.

15 As defined herein "effective amount" is in a range of about 50 ng to 10 mg. In one embodiment the effective amount may be up to 1 g. In another embodiment the effective amount is from about 2 to 50 μ g, more preferably the effective amount is 5 μ g. In a preferred embodiment, 5 μ g of peptide and 10 μ L of a suitable adjuvant are administered in a single injection. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual.

20 This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

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EXPERIMENTAL DETAILS**Materials and Methods:**

5 **Mice.** C57BL/6 (B6, H-2^b), B6D2 F₁ (H-2^b × ^d), and BALB/c (H-2^d) mice were obtained from the National Cancer Institute animal facility (Frederick, MD). B10.D2 (H-2^d) animals were purchased from Jackson Laboratories (Bar Harbor, ME). Animals of both sexes were used at 6-10 weeks of
10 age.

Peptides. Peptides were synthesized by the Memorial Sloan-Kettering Cancer Center (MSKCC) Microchemistry Core Facility using a standard f-moc method, followed by HPLC
15 purification and mass spectroscopy analysis, and were >98% pure, as evaluated by these methods. Sequences of peptides used in the study are shown in Table II.

Cell lines and viruses. MHC class II negative cell lines
20 EL-4 (H-2^b), its variants transfected with ovalbumin [18] and VSV nucleoprotein [12], P815 (H-2^d) and its variant transfected with listeriolysin O [11], and MC57 and its variant transfected with the Herpes Simplex virus glycoprotein B [19] were grown in RP10 [RPMI 1640
25 supplemented with antibiotics, 2-ME, glutamine, HEPES and 10% FBS (Gemini Bioproducts)]. Influenza virus strain A/PR8/34 (H1N1) [PR8 in the text], and VSV strain Indiana were obtained from Dr. M.J. Bevan (Univ. of Washington, Seattle, WA). HSV type I strain 17 was generously
30 provided by Dr. S. Silverstein (Columbia University, New York, NY). Indicated cell lines were infected with viruses in serum-free medium (RPMI 1640) at : (i) 10 pfu (plaque forming units)/cell for 1 h at 37°C for HSV, followed washing and a 5 hr incubation at 37°C; or (ii)
35 with 10⁻⁴ HAU (hemagglutinnin units) for 90' for PR8; or (iii) with 50 pfu VSV/cell, for 90'. For HSV infection, infected cells were then washed and labeled with ⁵¹Cr

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before the CTL assay. For PR8 and VSV, chromium labeling was performed simultaneously with infection. In vivo immunization with viruses was carried out by a single i.p. injection of 10^6 pfu/animal (VSV and HSV) or 300 HAU (influenza).

Immunization. Details of immunization protocols other than the one using Titermax® were described in references [1,5,9,10,13,14]. For peptide/Titermax® (pep/TM) immunizations, peptides were emulsified in Titermax® according to manufacturer's instructions, in the concentration of 0.5 mg/ml, and 10 μ l of this mixture injected s.c. into one footpad of methophane-anesthetized animals (unless otherwise indicated). This immunization did not appear to cause pain, discomfort, local inflammatory or allergic reactions. Non-anesthetized mice were also immunized without aberrant pain, stress, or discomfort [16].

Cytotoxic T Lymphocyte restimulation and ^{51}Cr -release assay. Six to eight days after restimulation, spleen cells from immunized animals were restimulated in 25-cm tissue culture flasks (Falcon, Becton-Dickinson, Mountain Viwe, CA), at $2-3 \times 10^7$ cells/flask in the presence of 2×10^7 peptide-coated irradiated (30 Gy) syngeneic spleen cells. Peptide coating was performed with 100 μ g of peptide/spleen, in HBSS without FBS, for 1h at 37°C, followed by three washes. Cultures were incubated at 37°C/5%CO₂, in a total volume of 10 ml of RPMI 1640 / 10% FCS (RP 10), with other supplements as described [10]. Cytotoxic activity of restimulated cultures was determined after five days in a classical 3-4 h ^{51}Cr -release assay. Target labeling, peptide coating and the assay were performed exactly as described [10], except that the standard peptide concentration for coating was 2 μ g/ml (unless indicated otherwise).

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Flow cytometric (FCM) analysis. Phenotype of cultured CTLs was determined using anti-CD8-FITC, anti-CD4-PE and anti-TcR β -FITC antibodies purchased from PharMingen (San Diego, CA). 10⁶ cells were simultaneously stained and analyzed for the expression of CD4⁺ and CD8⁺, or singly stained with anti-TcR β . After washing, cells were analyzed using a FACScan instrument and the LYSYS II software (Becton-Dickinson, Mountain View, CA). Control samples were stained with conjugated, species- and class-matched irrelevant antibodies (Fisher Biotech, Malvern, PA). Results from 10⁴ cells/sample are displayed as contour plots.

Antitumor activity of pep/TM-induced CTLs. For this assay, immunized or naive mice were irradiated with 4 Gy, anesthetized with methophane, their left flank shaven, and tumor injected s.c.. Mice were monitored daily for the tumor formation and the size of tumor measured by calipers. Mice were euthanized as soon as any of the following conditions were fulfilled: loss of motility, tumor ulceration, necrosis or inflammation, or tumor size of 25 mm.

Experiment 1. Induction of CTL Activity by Peptide Priming.

Recently, several groups induced CTL responses against purified or recombinant proteins of intracellular pathogens. These authors used vaccinia vectors [2] immunostimulatory complexes containing detergent components [18] or physical methods (electroporation) to deliver complex protein antigens into the cytosolic processing pathway [5], often by using several booster injections of the antigenic mixture. However, the production of such complex vaccines is usually elaborate, and the treatment could be complicated by multiple injections and the antigenicity of the whole protein.

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A preferable strategy would be to use smaller fragments of proteins to elicit CTL immunity, but the delivery of such fragments to class I molecules poses a problem. Indeed, CTL priming was recently achieved by peptides conjugated to lipid moieties [15] or emulsified in adjuvants [1] and in two instances it was shown that such immunization and subsequent boosting elicits CTLs that can protect against viral infection [7,8,17]. Peptides used for immunization were usually 15-16 residues long, and often contained not only a CTL but also a helper T lymphocyte (HTL) determinant. However, none of these protocols were tested with more than a single peptide and with more than one restricting MHC class I molecule. Potential advantages of shorter peptides would be : (i) simpler and cheaper synthesis; (ii) a lower probability to elicit unwanted (e.g. autoimmune) consequences; and (iii) an independence of their MHC class I binding on additional proteolytic processing.

In an attempt to elicit specific CTL responses in H-2K^b animals against the optimal ovalbumin peptide 257-264, SIINFEKL (OVA) (SEQ. ID. NO. 8), or VSV nucleoprotein peptide 52-59, RGYVYQGL (SEQ. ID. NO. 9) several protocols of immunization were tested in the same experiment. The only three priming protocols that yielded significant CTL activity specific for peptides were: thioglycolate-induced peritoneal macrophages coated with OVA, and peptide mixed with β_2 -microglobulin (β_2 -m), both as described by Rock et al. [13,14] and peptides emulsified in the synthetic adjuvant TiterMax®, abbreviated in the text as pep/TM (Table I). In subsequent experiments only pep/TM yielded reproducible results. Therefore CTL response to peptides in adjuvant was characterized, and tested to a broad range of peptides in order to evaluate the general features of this system.

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Initially priming was achieved with a single subcutaneous injection of a very low peptide amount (5 μ g). CTL response was vigorous and similar to that obtained in animals immunized with transfectants expressing the OVA determinant (Table I), or with spleen cells cytoplasmically "loaded" with the whole ovalbumin protein [9], two strategies commonly used to immunize with endogenously processed OVA. CTLs induced in this manner were propagated like conventional CTLs in a continuous culture by weekly restimulations with antigen and IL-2-rich Con A Sn; they were of CD8⁺4⁻ TcR $\alpha\beta$ ⁺ phenotype (Figure 1A and Figure 1B). Most importantly, the method was highly reproducible: CTLs were elicited in every single immunized B6 mouse, as shown in Table II (individual mice, and not pooled spleen cells were tested).

TABLE I. Methods of in vivo CTL priming by peptides

=====			
20	Immunization Protocol	Animals responding	% ⁵¹ Cr release
=====			
	E.G7 cells s.c.	2/2	74%, 49%
	Soluble protein i.v.	0/2	NA
25	Soluble protein/ 150 mM NaCl s.c	0/2	NA
	Soluble peptide i.v.	0/2	NA
	Soluble peptide s.c.	0/2	NA
	Peptide/IFA s.c.	0/2	NA
30	Peptide/CFA s.c.	0/2	NA
	Peptide/spleen i.v.	0/2	NA
	Peptide/spleen, s.c	0/2	NA
	Peptide/RMA-S s.c.	0/2	NA
	Peptide/TM s.c.	2/2	68%; 67%
35	Peptide/TM i.p.	1/2	71%
	Peptide/ β_2 -m s.c.	1/2	70%
	Peptide/Mp i.v.	1/2	40%
	Peptide/Mp s.c.	0/2	NA

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For Table 1. immunizations were performed in a single injection and at a single site. Wherever soluble peptide or peptide in adjuvant was used, the concentration of the peptide was 5 μ g/injection, in a total volume of 10 μ l. Emulsification in adjuvants was performed according to manufacturers' specifications. Coating of spleen cells, elicited macrophages (Mp), and RMA-S tumor cell lines was performed using 100 μ g of peptide and 10^8 cells for 1 h at 37°C, followed by three washes. β_2 -m was used at 10 μ g/injection, as described [13]. Seven days after immunization, splenocytes were restimulated with irradiated B6 spleen cells and EL-4 transfectants expressing endogenously processed OVA or VSV, in RPMI medium supplemented as described [10]. On day 5 of restimulation, cultures were tested for the presence of anti-OVA CTL activity using peptide-coated, 51 Cr-labeled EL-4 transfectant cells E.G7 (OVA) and N1 (VSV nucleoprotein), both H-2^b, class II⁻, as targets. Number of responding animals that displayed more than 20% transfectant-specific lysis is expressed as a fraction of total number of animals tested. % 51 Cr-release is displayed for responding animals. Lysis of control untransfected EL-4 cells was rather high, since these tumor cells were also used for restimulation in vitro, and varied from 10 to 35%.

Experiment 2. Peptide Priming as a General Method to Elicit Physiologically Relevant CTLs

To investigate whether this priming protocol may be effective for other peptides. Mice were immunized with four other K^b-restricted peptides, and one D^b-restricted peptide. Table III summarizes the obtained results. In every case potent CTL activity, specific for the immunizing, but not for irrelevant peptides, was obtained in each animal. The only exception was the H-2D^b-

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restricted influenza nucleoprotein peptide 366-374 (FLU-D^b), where three out of four animals mounted a good response, but the fourth responded weakly (~25% specific lysis). However, that line showed a substantial improvement of CTL activity upon the second in vitro restimulation. This subpar primary response could have been caused by a loss of priming mixture from the footpad due to leaking. An example of actual lysis by primary CTL cells is shown in Figure 2. Adjuvant alone did not elicit any CTL activity, whereas soluble peptide yielded low specific lysis, and CTLs from these cultures could not be propagated further (Figure 2).

CTLs against peptides were obtained previously, but were:

- (i) of low affinity, since they required high micromolar concentrations of antigen for target sensitization; and
- (ii) were specific for contaminating products in the peptide preparation, and not for the physiologically relevant peptides derived by endogenous processing and presentation [16].

Such CTLs were actually induced by peptide in vitro. By contrast, results shown in Table III. indicated that CTLs typically lysed target cells coated with as little as 10-100 pM of peptide. To test the specificity of CTL lines derived by peptide priming in vivo, ability of peptide-primed CTLs to lyse cell lines transfected with proteins from which the peptides were derived were examined (Table IV). CTL lines induced by pep/TM specifically lysed such transfectants, clearly showing that they are specific for physiologically processed peptides.

B6D2 F₁ mice were immunized with two optimal peptides derived from two different proteins of *L. monocytogenes*, [the major determinant of listeriolysin O (GYKDGNEYI (SEQ. ID. NO. 6) [5]) and the peptide derived from the secreted, invasion-related protein p60 (KYGVSVDI) (SEQ. ID. NO. 7), both restricted by H-2K^d. CTL activity

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obtained from these mice was strong and comparable to that obtained with H-2^b - restricted peptides (Figure 3). It was concluded that pep/TM method can be universally used with any MHC molecule and its corresponding optimal determinant(s).

TABLE II. Peptide-primed CTLs can be induced by a variety of viral peptides (SEQ. ID. NOS. 8-13)

=====				
10	Peptide	Origin	Sequence	# of responding animals/total animals immunized

	OVA	Ovalbumin	SIINFEKL	13/13
	VSV	VSV nucleoprotein	RGYVYQGL	16/16
15	SEN	Sendai nucleoprotein	FAPGNYPAL	2/2
	SVT	SV40 large T	VVYDFLKCL	6/6
	HSV	Herpes simplex gB	SSIEFARL	11/11
	FLU-D ^b	Influenza nucleoprotein	ASNENMETM	4*/4
=====				

20 For Table II. B6 mice were primed by a single footpad injection of 5 µg of indicated peptide in 10 µl of Titermax, and CTL activity assayed on peptide-coated EL-4 cells as described in the legend to Table I. Spontaneous lysis was < 20% of maximal, and all animals displayed

25 specific lysis levels between 40 and 70%. Lysis of EL-4 cells in the absence of peptide was <5%.

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TABLE III. Peptide-induced CTLs recognize target cells sensitized with picogram concentrations of peptide

CTL	Concentration of SEV peptide (ml ⁻¹) used to coat EL-4 targets					
	specificity	5 µg	50 ng	500 pg	5 pg	50 fg no peptide
SEV		68.3	69.7	72.1	56.4	4.9 4.7

For Table III. SEV-specific line LAG was derived by peptide priming and was maintained in culture by weekly restimulations as described. The line was tested against EL-4 cells coated with no peptide or with indicated concentrations of SEV peptide, and the results displayed as % specific ⁵¹Cr-release at the effector:target ratio of 25:1.

Experiment 3. Dose Dependency of the Priming

Efficient and reproducible priming with 5 µg of peptide in a single injection was obtained. To establish the limits of sensitivity of this priming protocol, priming dose was varied over several orders of magnitude for three peptides, OVA, VSV and FLU-D^b. Results of this assay are summarized in Table V. CTL αOVA response could be elicited by priming with as little as 0.5 µg/animal, although perhaps not as reproducibly as with higher doses. Anti-VSV response displayed similar dose dependency. However, FLU-D^b was effective only at a narrow range of concentrations, with optimal activity at 5 µg. Experiments with other peptides are in progress.

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TABLE IV. Peptide-primed CTL are specific for endogenously processed antigens

5	Priming peptide	<u>% specific ⁵¹Cr-release from targets</u>					
		EL-4	E.G7	N1	1308.1	MC57	MC57-gB
	TM alone	ND	ND	ND	ND	4.2	6.7
	HSV	ND	ND	ND	ND	5.1	79.9
10	VSV	0.6	1.1	80.0	ND	ND	ND
	SVT	4.0	ND	ND	28.0	ND	ND

For Table IV. peptide priming was performed as described above (Table. II) and CTL activity of anti-peptide cell lines tested on transfectants expressing endogenously processed peptide determinants. E.G7 and N1 are an EL-4 transfectants with ovalbumin and VSV nucleoprotein genes, respectively. 1308.1 is a H-2^b thymic epithelioma expressing the SV40 large T antigen, MC57 is a H-2^b fibrosarcoma, while MC57-gB has been transfected with the glycoprotein B of HSV.

TABLE V. Dose dependency of peptide priming

25	Priming peptide	<u># of responders/total after priming with</u>				
		50 µg	5 µg	500ng	50 ng	5ng
	OVA	3/3	3/3	2/3	0/3	0/3
30	VSV	3/3	3/3	3/3	0/3	0/3
	FLU-D ^b	0/2	2/2	0/2	0/2	0/2

For Table V. B6 mice were primed with indicated peptides in Titermax, and CTL activity of spleen cultures assayed after in vitro restimulation as described above. CTL activity higher than 25% of specific ⁵¹Cr-release for VSV and higher than 40% for OVA and FLU-D^b was considered

-24-

specific. This cutoff was confirmed by in vitro secondary stimulation of borderline cultures (those exhibiting 12-18% specific lysis), none of which gave any specific lysis upon re-testing. Lysis of EL-4 in the
 5 absence of peptides did not exceed 6% in any group.

Experiment 4. In vitro Antiviral Activity of Peptide-Induced CTLs

10 To test whether peptide-primed CTLs could be used to lyse target cells infected with intracellular pathogens, H-2^b cell lines with different viruses were infected, and tested against peptide-induced effector cells. For comparison, virus-induced CTLs were included in the assay
 15 in the case of HSV. The optimal HSV peptide induced CTLs that readily lysed virus-infected targets, and this antiviral activity was comparable to that of virus-induced CTLs (Table VI). Similar antiviral activity was obtained with FLU-D^b and VSV peptides (Table VI.).

20

TABLE VI. Antiviral activity of peptide-induced CTLs

=====				
Priming method	% specific ⁵¹ Cr-release from targets			
	MC57	MC57-gB	MC57/virus	

HSV Pep/TM	<1	74.9	48.2	
live HSV virus	<1	60.0	47.1	

	EL-4	EL-4/pept	N1	EL-4/virus
VSV pep/TM	2.4	56.1	81.2	59.3
=====				

For Table VI. B6 mice were immunized with the HSV glycoprotein B or VSV nucleoprotein peptide/Titermax[®] s.c., as described, or with 10⁶ pfu of HSV type 1, strain
 35 17, i.p.. After the establishment of long-term CTL lines by weekly in vitro restimulation (on peptide-coated spleen cell stimulators for peptide-induced CTLs or on

-25-

the transfectant in the presence of irradiated feeder cells for virus-induced CTLs), the CTL activity was tested on indicated targets described in legend to Table III. Results are displayed as the % specific ^{51}Cr -
5 release at effector:target ratio of 10:1.

Experiment 5. Antitumor CTL Responses Elicited by pep/TM

To test whether pep/TM strategy could be used to elicit
10 antitumor CTLs, a tumor model was established by injecting E.G7 thymoma cells s.c. into lightly irradiated syngeneic animals. Unirradiated animals reject this tumor in the course of 10-14 days, and allow only relatively small tumors (5-6 mm of diameter) to form at
15 any time between injection and rejection, even when relatively high doses of tumor cells (e.g. 10^7) are injected (Figure 4A). By contrast, upon light irradiation (4 Gy), mice injected with either 10^6 or 10^7 cells formed tumors (the former slightly slower than the
20 latter) that grew over 20 mm in diameter by 14 days. Owing both to the tumor size and appearance (necrosis, ulceration), animals were euthanized at this point.

Very different results were obtained when mice were
25 vaccinated by pep/TM using the OVA peptide, expressed at the surface of E.G7 cells. While control animals and animals receiving TM alone developed advanced tumors by day 7, and had to be euthanized by day 14 due to vigorous tumor growth, two out of three pep/TM vaccinated animals
30 did not develop tumors during that time at all, while the third animal developed a flat infiltration between days 4 and 7, the diameter of which could not be measured. The infiltration disappeared after day 7 (Figure 4B). These results reveal a considerable antitumor potential
35 of the pep/TM method.

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Experiment 6. Multiple Vaccination by pep/TM

To establish whether a cocktail of different peptides in TM can simultaneously elicit CTLs directed against each peptide in the mixture, mice were immunized with a cocktail containing OVA and SVT peptides (restricted by K^b) and a FLU peptide (restricted by D^b). Seven days later, spleen cells of immunized animals were divided in three, and restimulated with each of the three immunizing peptides separately. This protocol resulted in generation of strong and specific reactivity against each of the three peptides used in the immunizing cocktail (Figure 5). These results suggest that this method of immunization can be used to prime against several antigenic determinants on the same microorganism/tumor, or to prime simultaneously against several microorganisms/tumors.

Experiment 7. CTL Response to pep/TM is not Dependent on CD4⁺ T Cells

To test whether CD8⁺ CTL cells were dependent on CD4⁺ T cell help, animals deficient for class II molecules owing to a targeted disruption of the I-A_g gene were immunized. These mice have a tenfold reduction in CD4⁺ T cell numbers. To our surprise, these mice mounted a CTL response to HSV and SVT peptides indistinguishable from that of control B6 mice. Given that these mice still contain 5-10% of the normal CD4⁺ cell numbers, it was still possible that these cells were essential for the CTL response. Therefore CD4⁺ cells from normal mice were depleted by in vivo mAb treatment (GK 1.5 mAb, 50 μ l of purified mAb on days -1, 2 and 5). Animals treated in this way contained less than 0.5% CD4⁺ cells in the peripheral blood at the time of immunization and in the spleen at the time of sacrifice. However, CTL responses were intact in these animals (Figure 6). These results

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indicate that pep/TM induces CD4⁺-independent CTLs, and indicate that this method could be successfully used in situations where CD4⁺ T cells are immunocompromised or scarce, such is the case with HIV infections.

5

Discussion:

By using peptides to elicit CTLs one can vaccinate mammals against all three classes of intracellular microorganisms (viruses, bacteria and parasites), and, as demonstrated here, against malignant tumors. A prerequisite for the successful application of this method is the knowledge of the relevant peptide. Several methods can be used to identify such peptides as immunodominant CTL determinants [3,4] and it is possible to identify these peptides out of tumor cells or infected cells in humans [4]. Once the peptides are identified, they can be mixed with a suitable adjuvant, and used to vaccinate animals and humans. Therefore, peptide vaccination should provide a simple general method of eliciting CTL immunity.

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5

-30-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Nikolic-Zugic, Janko
Dyall, Rubendra
- (ii) TITLE OF INVENTION: INDUCTION OF CYTOTOXIC T LYMPHOCYTES (CTL) USING
ANTIGENIC PEPTIDES AND A SUITABLE ADJUVANT
- (iii) NUMBER OF SEQUENCES: 19
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Cooper & Dunham LLP
 - (B) STREET: 1185 Avenue of the Americas
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.24
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/233,496
 - (B) FILING DATE: April 22, 1994
 - (C) CLASSIFICATION:
- (v) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: White Esq., John P.
 - (B) REGISTRATION NUMBER: 28,678
 - (C) REFERENCE/DOCKET NUMBER: 45059/JPW/MS/AMB
- (x) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 212-278-0400
 - (B) TELEFAX: 212-391-0525

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: N
- (iv) ANTI-SENSE: N
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
Glu Val Asp Pro Ile Gly His Leu Tyr
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(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:

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-31-

(A) LENGTH: 9 amino acids
(B) TYPE: amino acids
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acids
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

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(A) LENGTH: 9 amino acids
(B) TYPE: amino acids
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

-32-

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Leu Thr Ser Cys Asn Thr Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Tyr Lys Asp Gly Asn Glu Tyr Ile
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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Tyr Gly Val Ser Val Gln Asp Ile
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Ile Ile Asn Phe Glu Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:9:

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-33-

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: N
- (vi) ANTI-SENSE: N
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
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- (2) INFORMATION FOR SEQ ID NO:10:
- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: N
- (vi) ANTI-SENSE: N
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
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- (2) INFORMATION FOR SEQ ID NO:11:
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 (B) TYPE: amino acid
 (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: N
- (vi) ANTI-SENSE: N
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
 Val Val Tyr Asp Phe Leu Lys Cys Leu
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- (2) INFORMATION FOR SEQ ID NO:12:
- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: N

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(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ser Ser Ile Glu Phe Ala Arg Leu
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Ile Leu Gly Phe Val Phe Pro Leu
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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Leu Phe Gly Tyr Pro Val Tyr Val
1 5

-35-

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ile Leu Lys Glu Pro Val His Gly Val
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(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Lys Leu Gly Glu Phe Tyr Asn Gln Met Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ile Ala Gly Asn Ser Ala Tyr Glu Tyr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

-36-

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Phe	Leu	Ala	Ser	Asp	Phe	Phe	Pro	Ser	Val
1				5					10

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What is claimed is:

1. A method of treating a subject with a tumor which comprises administering to the subject an effective amount of a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the tumor.
2. The method of claim 1, wherein the suitable adjuvant is "TITERMAX".
3. The method of claim 1, wherein the effective amount the MHC class I restricted 8-12 amino acid antigenic peptide in combination with the effective amount of a suitable adjuvant is administered in combination with a second anti-tumor therapy.
4. A method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby induce cytotoxic T lymphocytes in the subject.
5. The method of claim 4, wherein the cytotoxic T lymphocyte is an anti-tumor cytotoxic T lymphocyte.
6. The method of claim 4, wherein the suitable adjuvant is "TITERMAX".
7. A method of treating a subject with a pathogenic disease which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to

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thereby treat the subject with the pathogenic disease.

- 5 8. The method of claim 7, wherein the suitable adjuvant is "TITERMAX".
9. The method of claim 7, wherein the pathogenic disease is bacterial.
- 10 10. The method of claim 7, wherein the pathogenic disease is parasitic.
11. The method of claim 7, wherein the MHC class I restricted 8-12 amino acid antigenic peptide is
15 selected from the group consisting of EVDPIGHL, EADPTGHSY, EVVPISHLY, EIRSLYNPV, PLTSCNTSV, GYKDGNEYI, KYGVSVQDI, SIINFEKL, RGYVYQGL, FAPGNYPAL, VVYDFLKCL, SSIEFARL, ASNENMETM, GILGFVFPPL, LLFGYPVYV, ILKEPVHGV, KLGEFYNQMM, IAGNSAYEYV,
20 FLASDFFPSV (SEQ. ID. NOS. 1-19).
12. A method of vaccinating a subject which comprises administering to the subject an effective amount of
25 a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby vaccinate the subject.
13. A method of inducing cytotoxic T lymphocytes in a
30 subject which comprises administering to the subject an effective amount of a MHC class I restricted amino acid antigenic peptide in combination with an effective amount of "TITERMAX" so as to thereby induce cytotoxic T lymphocytes in the subject.
- 35 14. The method of claim 1 or 7, wherein a plurality of antigenic peptides are administered in combination

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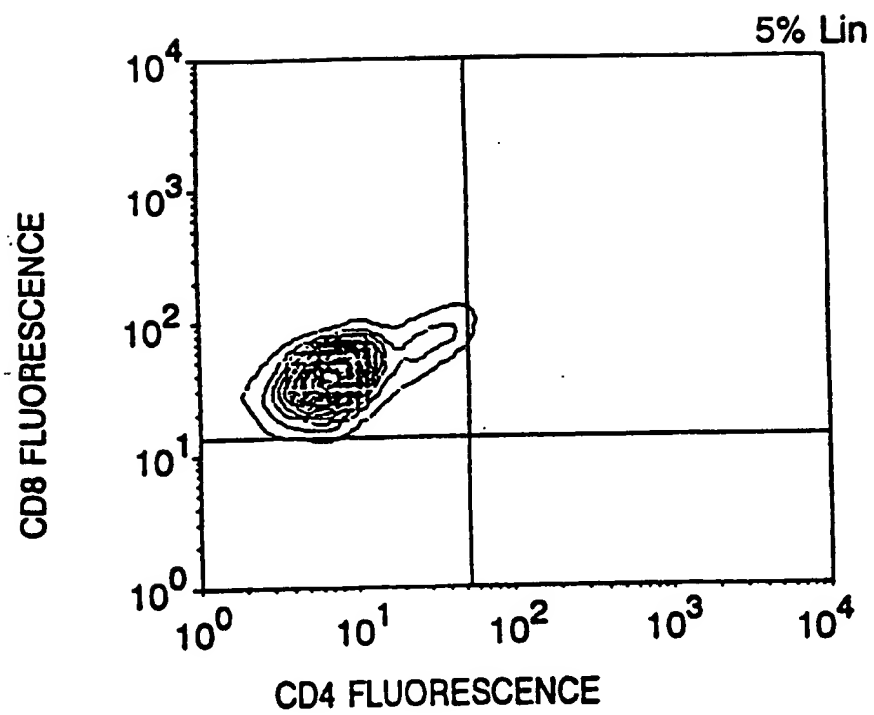
with an effective amount of the suitable adjuvant.

15. The method of claim 1 or 7, wherein the subject is a mammal.
- 5 16. The method of claim 15, wherein the mammal is selected from a group consisting of a human, monkey, dog, cow, horse, chicken or rodent.
- 10 17. The method of claim 1 or 7, wherein the administration is intratumorally, intradermal, topical, oral, intravenous, intramuscular, intratracheal, or by subcutaneous administration.
- 15 18. The method of claim 1 or 7, wherein the effective amount is a range of about 500 ng to about 10 mg.
19. The method of claim 18, wherein the effective amount is in a range of about 2 μ g to 50 μ g.
- 20 20. A kit for inducing cytotoxic T lymphocytes in a subject which comprises: a suitable amount of MHC class I restricted 8-12 amino acid antigenic peptide and a suitable adjuvant.
- 25 21. The method of claim 20, wherein the suitable adjuvant is "TITERMAX".
- 30 22. The method of claim 20, wherein the MHC class I restricted 8-12 amino acid antigenic peptide is selected from the group consisting of EVDPIGHL, EADPTGHSY, EVVPISHLY, EIRSLYNPV, PLTSCNTSV, GYKDGNEYI, KYGVSVDI, SIINFEL, RGVYQGL, FAPGNYPAL, VVYDFLKCL, SSIEFARL, ASNENMETM, GILGFVFP, LLFGYPVYV, ILKEPVHGV, KLGEFYNQMM, IAGNSAYEYV, FLASDFFPV (SEQ. ID. NOS. 1-19).
- 35

SUBSTITUTE SHEET (RULE 26)

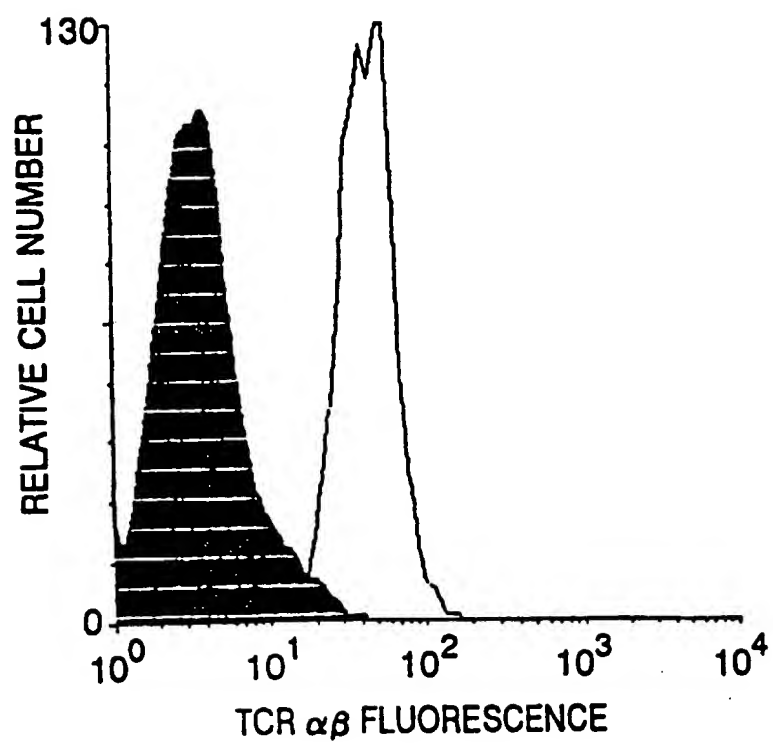
1/8

FIGURE 1A

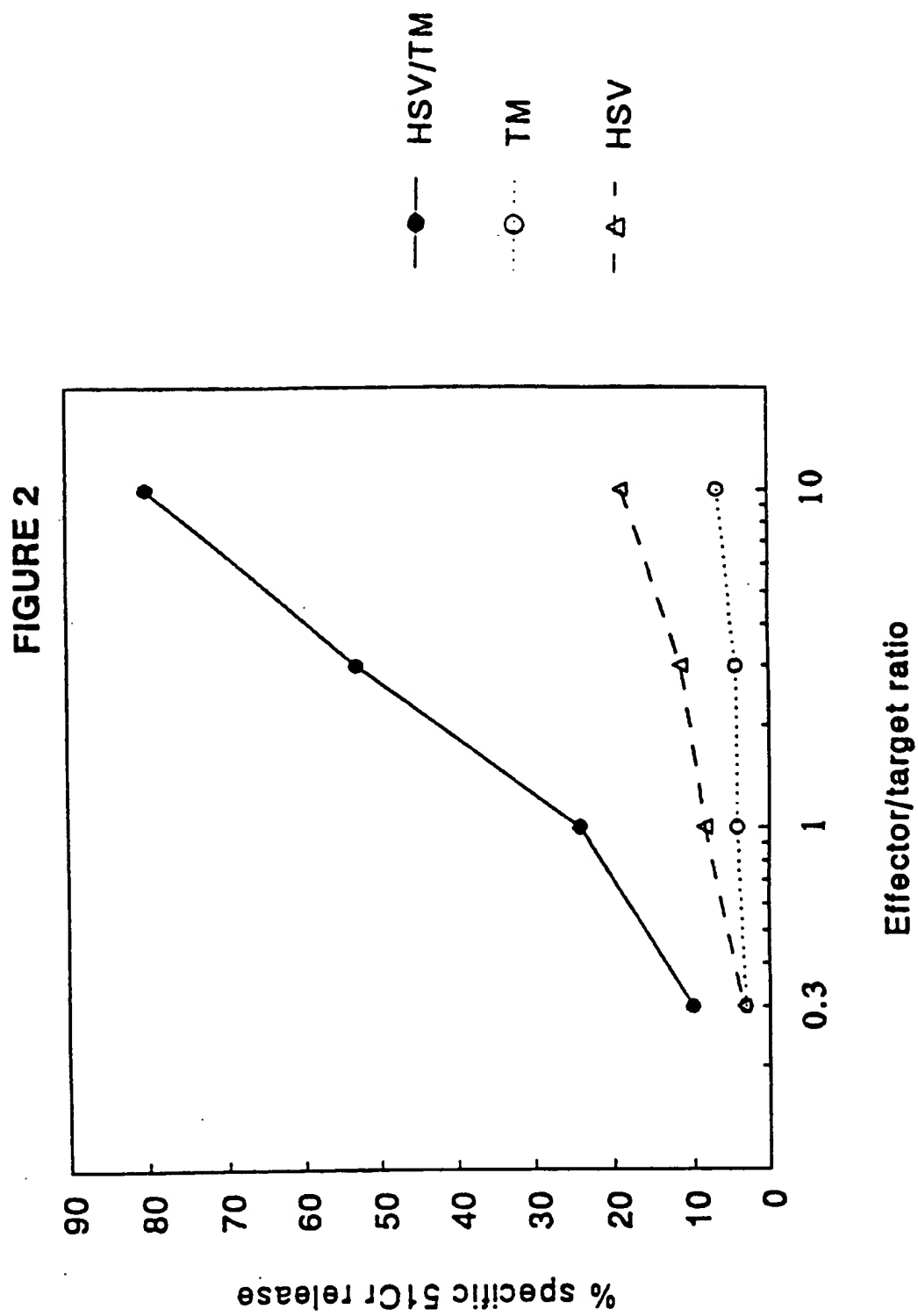


2/8

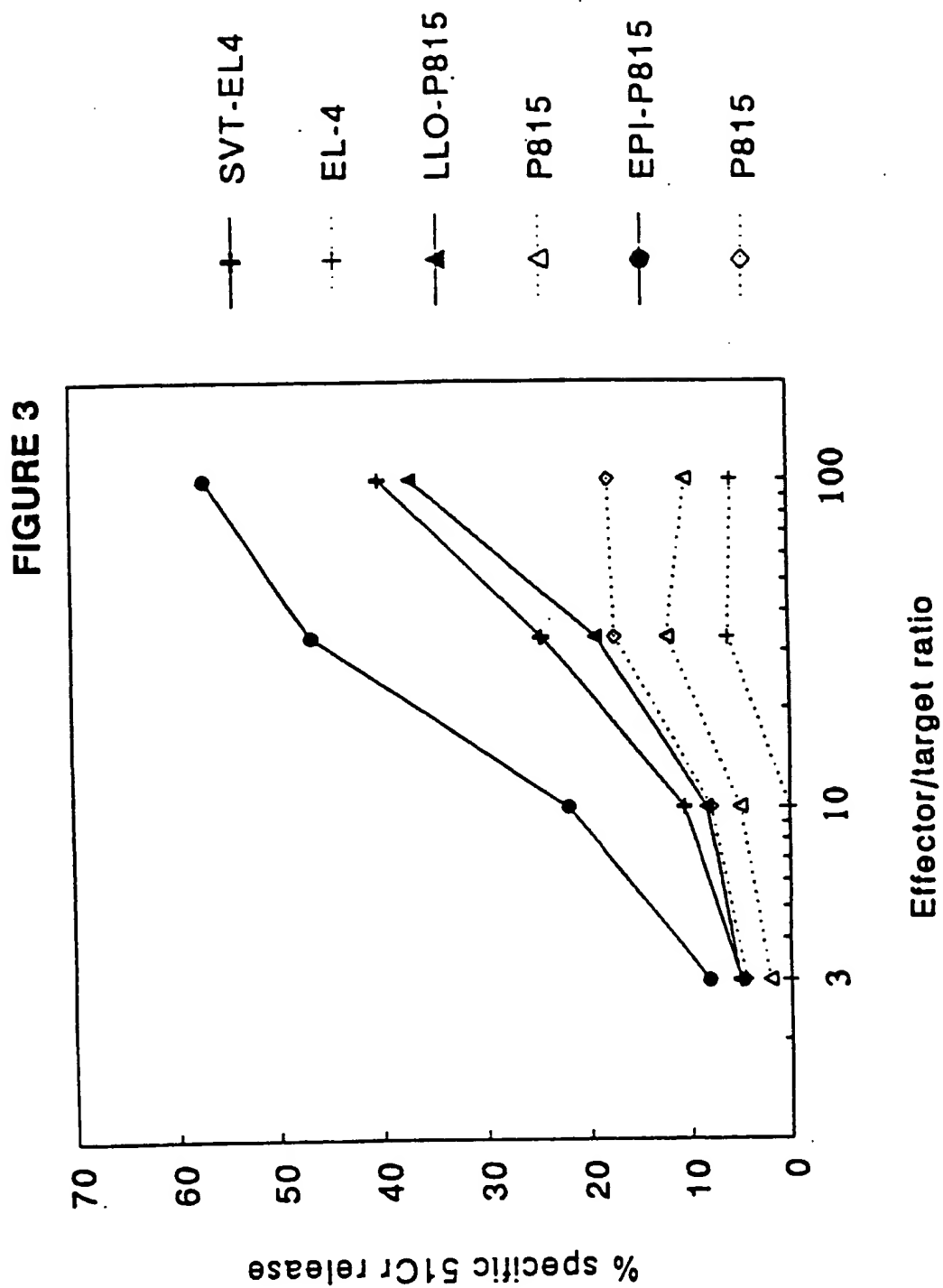
FIGURE 1B



3/8

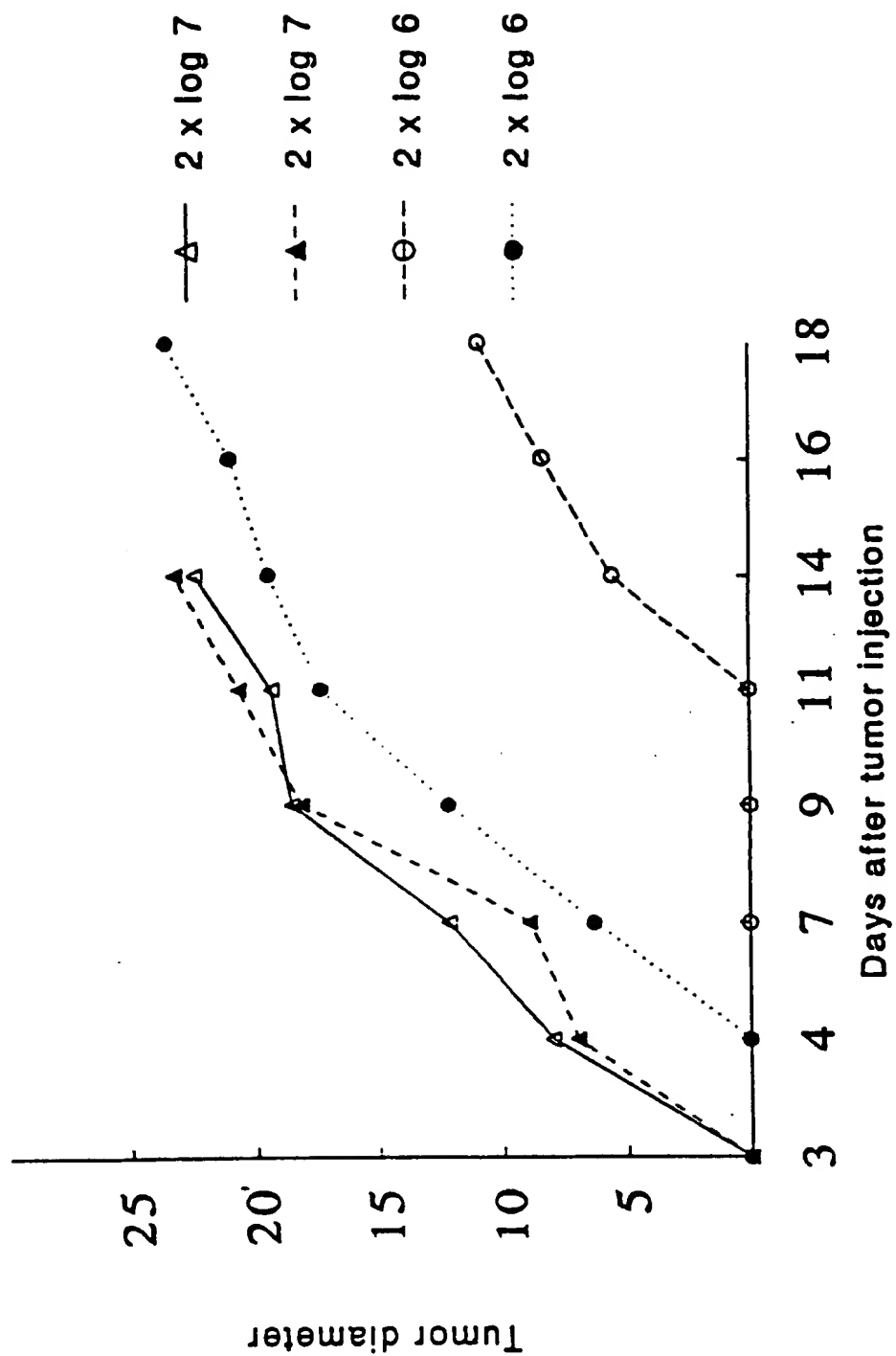


4/8



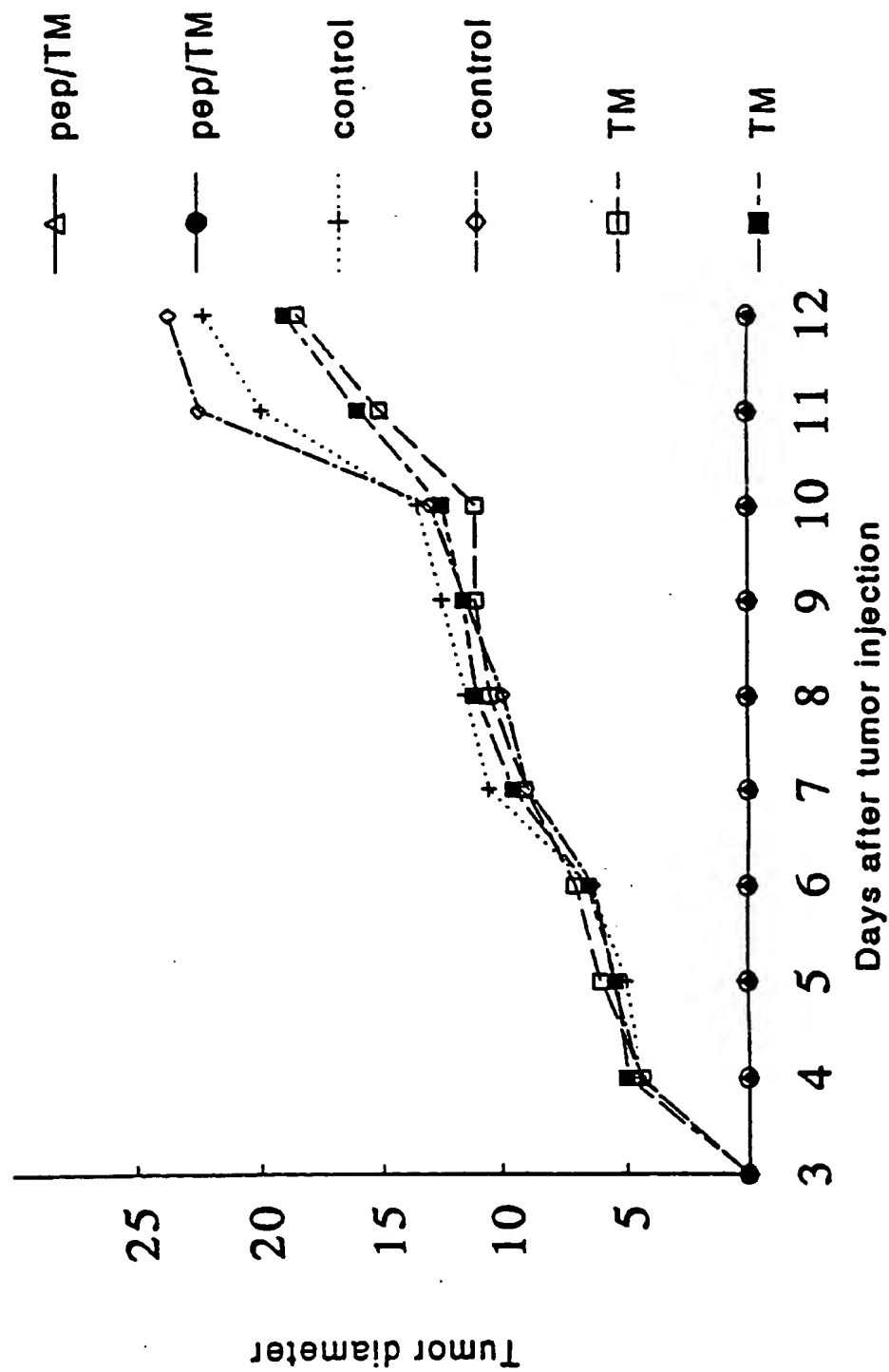
5/8

FIGURE 4A



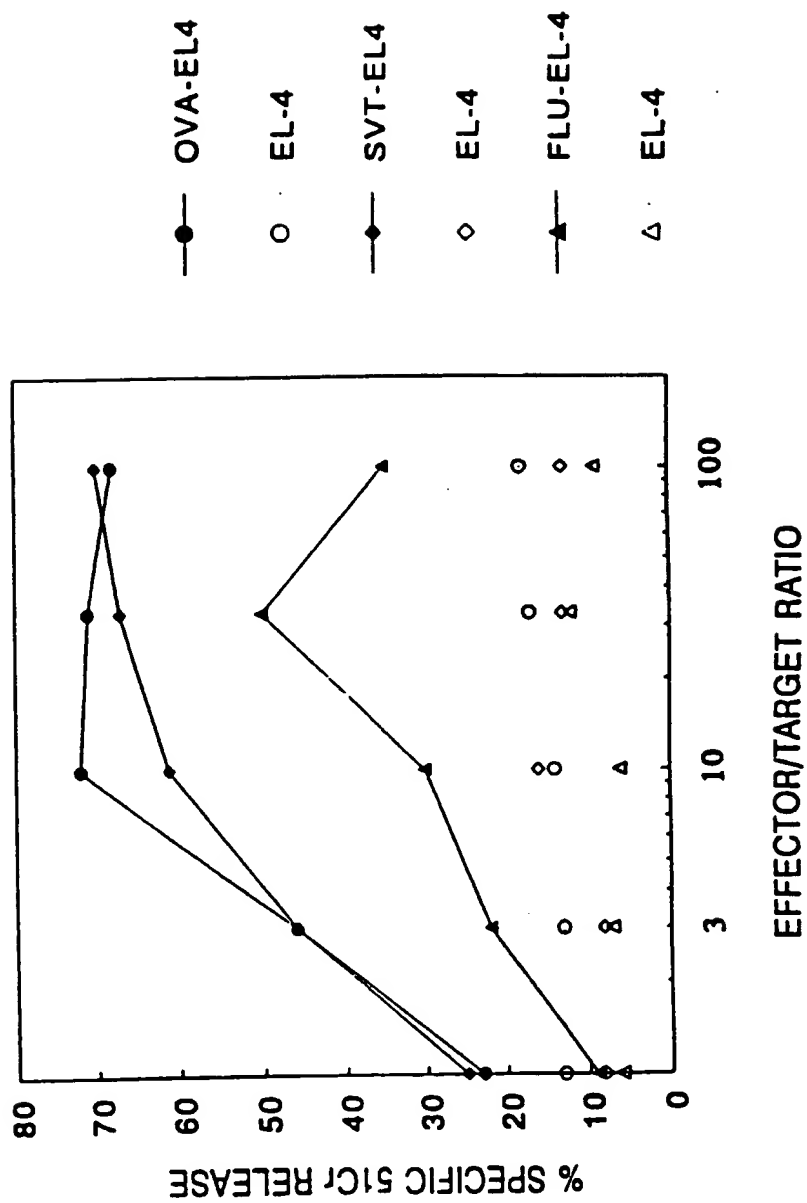
6/8

FIGURE 4B



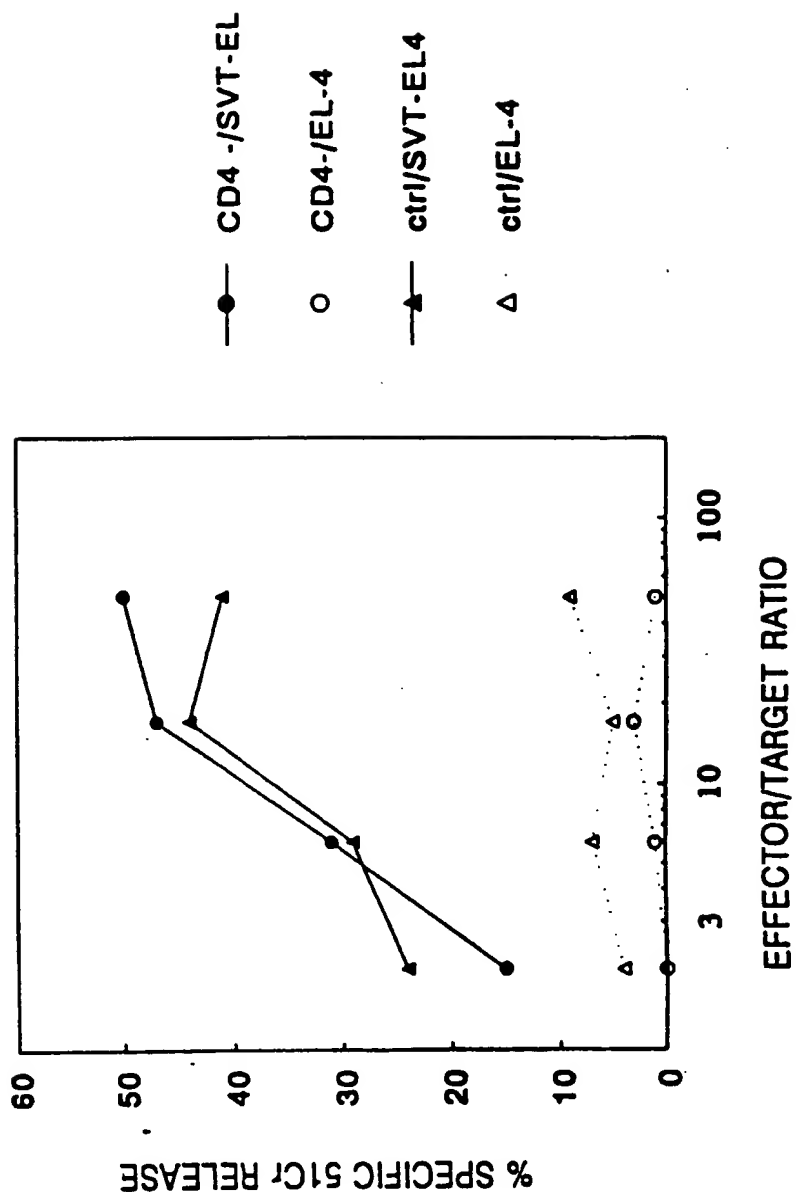
7/8

FIGURE 5



8/8

FIGURE 6



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/04975

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/00, 39/385, 39/39; C07K 14/74

US CL : 424/184.1, 185.1, 277.1, 278.1; 530/300, 350, 395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 185.1, 277.1, 278.1; 530/300, 350, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent System (APS) and DIALOG (file BIOCHEM) databases. Key words: MHC Class I, tumor, cancer, peptide.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,478,823 (SANDERSON) 23 October 1984, see the claims.	1-22
A	US, A, 5,130,297 (SHARMA ET AL.) 14 July 1992, see entire document.	1-22
Y	W. F. Paul, "Fundamental Immunology", 3rd edition, published by Raven Press (N.Y.), pages 615-628, see entire section.	1-22
Y	Cancer Research, vol. 53, issued 01 January 1993, Zakut et al., "Differential expression of MAGE-1, -2, and -3 messenger RNA in transformed and normal human cell lines", pages 5-8, see entire article.	1-22
Y	US, A, 5,200,320 (SETTE ET AL.) 06 April 1993, see entire document.	1-22

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 JULY 1995

Date of mailing of the international search report

28 JUL 1995

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

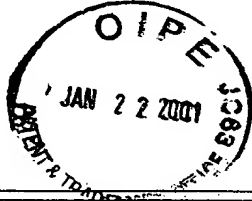
THOMAS M. CUNNINGHAM

Telephone No. (703) 308-0196

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: MARINA T. LARSON
OPPEDAHL & LARSON LLP
611 MAIN STREET
P.O. BOX 5540
FRISCO, CO 80443-5540



PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing
(day/month/year)

29 DEC 2000

Applicant's or agent's file reference

MSK.P-042WO

IMPORTANT NOTIFICATION

International application No.

PCT/US99/13146

International filing date (day/month/year)

11 JUNE 1999

Priority Date (day/month/year)

12 JUNE 1998

Applicant

SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH

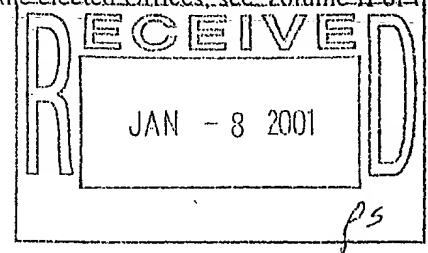
1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

REPORTING LETTER _____
COMPUTER DOCKET _____
PAPER DOCKET _____



Name and mailing address of the IPEA/US

Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARIANNE DIBRINO

Marianne Dibrino

Telephone No. (703) 308-0196

PATENT COOPERATION TREATY

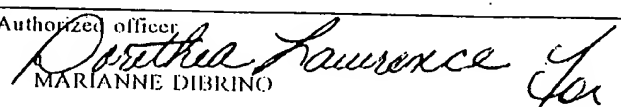
PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference MSK.P-042WO	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/13146	International filing date (day/month/year) 11 JUNE 1999	Priority date (day/month/year) 12 JUNE 1998
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 38/00, 38/04, 38/08, 38/10 and US Cl.: 514/14, 15, 16, 44		
Applicant SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH		

<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of <u>6</u> sheets.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of <u>0</u> sheets.</p> <p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input checked="" type="checkbox"/> Non-establishment of report with regard to novelty, inventive step or industrial applicability IV <input checked="" type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application
--

Date of submission of the demand 16 DECEMBER 1999	Date of completion of this report 13 AUGUST 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer  MARIANNE DIBRINO Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

I. Basis of the report

1. With regard to the **elements** of the international application:*

- ☒ the international application as originally filed
- ☒ the description:
pages 1-22 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____
- ☒ the claims:
pages 23-27 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____
- ☒ the drawings:
pages 1-9 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____
- ☒ the sequence listing part of the description:
pages 1-7 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in printed form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

- ☒ the description, pages NONE
- ☒ the claims, Nos. NONE
- ☒ the drawings, sheets/fig NONE

5. ☒ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

**Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/13146

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. (Please See supplemental sheet)

because:

- ☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination (*specify*).

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _ are so unclear that no meaningful opinion could be formed (*specify*).

- ☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

- ☒ no international search report has been established for said claims Nos. (See Attached).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
- ☒ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- ☐ complied with.
- ☒ not complied with for the following reasons:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-4 and 6-30 (claims 17-23 and 25 in part as they pertain to the peptide immunogen of Group I and the species peptide derived from gp75) and 32-33 (as they pertain to the peptide immunogen of Group I and the species peptide derived from gp75), drawn to a method of inducing a cellular immune response using a peptide immunogen, a method for preparation of a peptide vaccine and a peptide immunogen and composition, thereof.

Group II, claims 1, 5, 6 and 11-16, drawn to a method for inducing a cellular immune response by administration of a nucleic acid encoding a peptide (as Group II claims pertain to the species peptide derived from gp75).

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- ☐ all parts.
- ☐ the parts relating to claims Nos. .

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. statement**

Novelty (N)	Claims	<u>1-14, 17-23, 25, 27-30, 32, 33</u>	YES
	Claims	<u>NONE</u>	NO
Inventive Step (IS)	Claims	<u>1-14, 17-23, 25, 27-30, 32, 33</u>	YES
	Claims	<u>NONE</u>	NO
Industrial Applicability (IA)	Claims	<u>1-14, 17-23, 25, 27-30, 32, 33</u>	YES
	Claims	<u>NONE</u>	NO

2. citations and explanations (Rule 70.7)

Applicant's response to the written opinion filed 7/11/00 has been considered with the following effect. Applicant's argument on inherency with regard to Kawakami et al (WO 95/29193) is found persuasive.

Claims 1-14, 17-23, 25, 27-30, 32 and 33 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the claimed methods for inducing a cellular response using non-immunogenic or weakly immunogenic peptides altered to have higher binding affinity to MHC, the said peptides and vaccine compositions thereof, or methods of preparing said vaccine compositions. Said peptides, compositions and methods are useful for inducing immune responses to tumor antigens.

----- NEW CITATIONS -----

KAWAKAMI et al. Identification of the Immunodominant Peptides of the MART-1 Human Melanoma Antigen Recognized by the Majority of HLA-A2-restricted Tumor Infiltrating Lymphocytes. J. Exp. Med. July 1994, Vol. 180, pages 347-352, see entire document, especially Abstract.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

I. BASIS OF REPORT:

5. (Some) amendments are considered to go beyond the disclosure as filed:
NONE

III. NON-ESTABLISHMENT OF REPORT:

The questions of whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect to claim numbers 17-26 and 31 in part as they pertain to a nucleotide and claims 15, 16, 24 and 26 (species not paid for).

No international search report has been established for claim numbers 17-26 and 31 in part as they pertain to a nucleotide and claims 15, 16, 24 and 26 (species not paid for).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 05 JAN 2001

WIPO

PCT

Applicant's or agent's file reference MSK.P-042WO	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/13146	International filing date (day/month/year) 11 JUNE 1999	Priority date (day/month/year) 12 JUNE 1998
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 38/00, 38/04, 38/08, 38/10 and US Cl.: 514/14, 15, 16, 44		
Applicant SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH		

- This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
- This REPORT consists of a total of 6 sheets.
☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).
These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

**CORRECTED
VERSION**

Date of submission of the demand 16 DECEMBER 1999	Date of completion of this report 13 AUGUST 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer <i>Marianne Dibrino</i> MARIANNE DIBRINO
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

I. Basis of the report

1. With regard to the elements of the international application:*

- ☒ the international application as originally filed
- ☒ the description:
pages 1-22, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the claims:
pages 23-27, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the drawings:
pages 1-9, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the sequence listing part of the description:
pages 1-7, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in printed form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

- ☒ the description, pages NONE
- ☒ the claims, Nos. NONE
- ☒ the drawings, sheets/Fig NONE

5. ☒ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

**Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. (Please See supplemental sheet)

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination (*specify*).

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _ are so unclear that no meaningful opinion could be formed (*specify*).

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. (See Attached).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
- ☒ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- ☐ complied with.
- ☒ not complied with for the following reasons:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

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4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- ☐ all parts.
- ☐ the parts relating to claims Nos. .

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. statement**

Novelty (N)

Claims 1-14, 17-23, 25, 27-30, 32, 33 YESClaims NONE NO

Inventive Step (IS)

Claims 1-14, 17-23, 25, 27-30, 32, 33 YESClaims NONE NO

Industrial Applicability (IA)

Claims 1-14, 17-23, 25, 27-30, 32, 33 YESClaims NONE NO**2. citations and explanations (Rule 70.7)**

Applicant's response to the written opinion filed 7/11/00 has been considered with the following effect. Applicant's argument on inherency with regard to Kawakami et al (WO 95/29193) is found persuasive.

Claims 1-14, 17-23, 25, 27-30, 32 and 33 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the claimed methods for inducing a cellular response using non-immunogenic or weakly immunogenic peptides altered to have higher binding affinity to MHC, the said peptides and vaccine compositions thereof, or methods of preparing said vaccine compositions. Said peptides, compositions and methods are useful for inducing immune responses to tumor antigens.

----- NEW CITATIONS -----

KAWAKAMI et al. Identification of the Immunodominant Peptides of the MART-1 Human Melanoma Antigen Recognized by the Majority of HLA-A2-restricted Tumor Infiltrating Lymphocytes. J. Exp. Med. July 1994, Vol. 180, pages 347-352, see entire document, especially Abstract.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/13146

Supplemental B x

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

I. BASIS OF REPORT:

5. (Some) amendments are considered to go beyond the disclosure as filed:

NONE

III. NON-ESTABLISHMENT OF REPORT:

The questions of whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect to claim numbers 17-26 and 31 in part as they pertain to a nucleotide and claims 15, 16, 24 and 26 (species not paid for).

No international search report has been established for claim numbers 17-26 and 31 in part as they pertain to a nucleotide and claims 15, 16, 24 and 26 (species not paid for) .